(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 797 993 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
01.10.1997 Bulletin 1997/40

(21) Application number: **97103727.0**

(22) Date of filing: **06.03.1997**

(51) Int. Cl.[6]: **A61K 31/205**, A61K 31/22, A61K 31/095, A61K 31/375, A61K 31/07, A61K 31/355

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **29.03.1996 IT RM960199**

(71) Applicant: **Sigma-Tau Industrie Farmaceutiche Riunite S.p.A.**
00144 Roma (IT)

(72) Inventor: **Cavazza, Claudio**
Roma (IT)

(74) Representative: **Fassi, Aldo, Dr,**
c/o CON LOR SPA,
Via Renato Fucini, 14
20133 Milano (IT)

(54) COMPOSTIONS COMPRISING L-CARNITINE OR DERIVATIVES THEREOF AND ANTIOXIDANTS

(57) Therapeutical use is described of L-carnitine, some alkanoyl L-carnitines and the pharmacologically acceptable salts thereof in combination with hydrophilic antioxidants or lipophilic antioxidants or derivatives thereof or natural products containing said substances for the prevention and treatment of deseases of the central nervous system such as parkinsonism, trauma, cerebral palsy, diabetic neuropathy; of the peripheral nervous system such as diabetic peripheral neuropathy and traumatic nerve damage; of the cardiovascular system such as stroke ischaemic-reperfuson damage and intermittent claudication; and immune system abnormalities in conditions of low oxygen tension.

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Printed by Rank Xerox (UK) Business Services
2.14.14/3.4

Description

The present invention relates to a novel terapeutic use of L-carnitine, some alkanoyl L-carnitines and the pharmacologically acceptable salts thereof for the prevention and treatment of disturbances and diseases elicited by the oxidative stress brought about by oxygen free radicals.

More specifically, the present invention relates to the coordinated use of L-carnitine or an alkanoyl L-carnitine or the pharmacologically acceptable salts thereof with natural lipophilic antioxidants such as vitamin E or vitamin A and/or natural hydrophilic antioxidants such as vitamin C, glutathione (GSH) or selenium.

Several therapeutic uses of both L-carnitine and alkanoyl L-carnitine are already known, none of which Is related to the use disclosed herein.

According to the present invention, by "co-ordinated use" of the aforesaid compounds it is meant indifferently either the co-administration, i.e. the substantially concomitant supplementation of L-carnitine, alkanoyl L-carnitine or a pharmacologically acceptable salt thereof and a natural lipophilic antioxidant such as vitamin E or vitamin A, or a natural hydrophilic antioxidant such as vitamin C, GSH or selenium, or the administration of a combination preparation or admixture of the aforesaid active ingredients, in addition to suitable excipients, if any.

Therefore, the present invention also relates to orally, parenterally, rectally or transdermally administrable pharmaceutical compositions suitable for treating disorders and pathologies related to the oxidative stress to body proteins and fatty acids which comprise, as active ingredients, L-carnitine, an alkanoyl L-carnitine or a pharmacologically acceptable salt thereof and a natural lipophilic antioxidant such as vitamin E or vitamin A and/or a natural hydrophilic antioxidant such as vitamin C, GSH or selenium.

The disturbances and diseases associated with protein oxidation include central nervous system diseases such as parkinsonism, trauma, cerebral palsy, diabetic neuropathy and ageing; peripheral nervous system diseases such as diabetic peripheral neuropathy and traumatic nerve damage; diseases of the cardiovascular system such as intermittent claudication, ischaemic-reperfuson damage and stroke; and immune system abnormalities in conditions of low oxygen tension.

Though it has long been postulated that the formation of free radicals is the probable cause of ischaemic damage, it has proved hard to demonstrate directly that such formation occurred and/or that it was sufficiently pronounced to overcome the antioxidative defences of the tissues, as reported by Curran et al., *Mol. Cell. Biol.* **5**, 167-172, 1985.

In many cases the oxygenated tissues undergo damage, which may even be permanent, if they become ischaemic and are then reperfused.

Molecular oxygen reduction products (MORP) are regarded as being responsible for cell damage in the course of ischaemia and postischaemic reperfusion of organs such as the brain, heart, intestines and kidneys [Braughler, J.M. and Hall, E.D. (1989) *Free Rad. Biol. Med.* **6**, 289-301].

Moreover, in inflammation, ageing and other important biological processes MORP are known to be mediators and/or modulators of various cell responses [Turner, E. et al., (1988) *Science* **242**, 939-941].

Several therapeutic protocols have been developed aimed mainly at eliminating the toxic effect of MORP on target biological structures such as the cell membrane. The most commonly adopted approach has been to use molecules which mimic the well-known antioxidant action of natural compounds, which constitute part of the so-called primary antioxidant front [Bolli, R. (1989) *J. Am. Coll. Cardiol.* **12**, 239-249]. The most commonly used substances are, for example, superoxide dismutase, an enzyme capable of specifically removing the superoxide anion, and vitamin E, a molecule belonging to the tocopherol family capable of interrupting dangerous oxidative reactions affecting polyunsaturated fatty acids. Recent years have witnessed the marketing of OTC products, in which various combinations of non-enzymatic antioxidants (vitamin E, vitamin A, vitamin C, selenium, glutathione, etc.) are present. Clearly, this type of approach tends exclusively towards the removal of MORP or of other reactive molecules which are produced by the interaction of MORP with macromolecules of biological interest. This strategy takes no account of those biological abnormalities which set in even when it proves possible to reduce the original toxic potential of MORP. Furthermore, pathological events of unpredictable onset, such as organ ischaemia, often do not allow effective therapeutic intervention designed to cope with the antioxidant action of MORP. Thus, it is necessary to sensitize and/or potentiate the cell repair mechanisms in order to minimize the toxic effects due to the damaging action of MORP.

The exclusion of a direct antioxidizing action, i.e. of the primary type, of L-carnitine and its esters against MORP has already been abundantly demonstrated [Arduini, A., et al. (1990) *Free Rad. Res. Commun.* **10**, 325-332].

The typical target of MORP in the course of oxidative stress is the plasma membrane, whose integrity is essential for cell survival. The polyunsaturated fatty acids of membrane phospholipids are particularly sensitive to the oxidizing action of MORP. These fatty acids are capable of propagating the peroxidative reactions triggered by MORP, thus stabilizing the intermediate radical species [Mead, J.F. (1976) in "*Free Radicals in Biology*" (Pryor W.A. ed.) Vol. 1 pp. 51-80, Academic Press, New York]. The peroxidation of the biological membranes may generate various oxidation products which cause significant alterations of membrane structure and function [Arduini, A. et al. (1989) *Arch. Biochem. Biophys.*, **273**, 112-119].

The invention described herein is based on the sur-

prising synergistic effect occurring between L-carnitine, or an alkanoyl L-carnitine (as it will be specified hereinbelow) or a pharmacologically acceptable salt thereof and a lipophilic natural antioxidant such as vitamin E or vitamin A and/or a hydrophilic natural antioxidant such as vitamin C, GSH or selenium. This synergistic effect is particularly surprising on account of the above-mentioned lack of direct antioxidizing action of L-carnitine and its aforesaid derivatives.

It has now been found that the coordinated use of L-carnitine, an alkanoyl L-carnitines or the pharmacologically acceptable salts thereof and the aforesaid antioxidant agents, whilst eliminating the toxic effect elicited by MORP's against the targeted biological structures of cells, not only prevents cell damage but also enhances the process of cell repair, thus allowing remarkable therapeutic results to be achieved.

The alkanoyl L-carnitines useful for the novel therapeutical use of the present invention are those wherein the alkanoyl group is a straight or branched group having from 2 to 8, preferably from 2 to 6 carbon atoms.

Particularly preferred are acetyl, propionyl, butyryl, valeryl and isovaleryl L-carnitine.

Pharmaceutically acceptable salts of carnitine or alkanoyl L-carnitine include, in addition to the inner salts, all the pharmaceutically acceptable salts which are prepared by the addition of an acid to L-carnitine, respectively, and which do not give rise to undesirable toxic or side effects.

The formation of pharmaceutically acceptable acid addition salts is well known to the experts in pharmacy and pharmaceutical technology.

Non-limiting examples of suitable salts include the chloride, bromide, orotate, acid aspartate, acid citrate, acid phosphate, fumarate, acid fumarate, lactate, maleate, acid maleate, acid oxalate, acid sulphate, glucose phosphate, tartrate and acid tartrate salts.

For the sake of simplicity and clarity, hereinbelow reference will be made to L-carnitine only, it being understood, however, that whatever disclosed in connection with L-carnitine equally applies to the above-identified alkanoyl L-carnitines and pharmacologically acceptable salts thereof.

The compositions of the present invention prove particularly effective in inhibiting the toxic effect of MORP by acting both as primary and secondary antioxidants with the result that they can be used in the pharmaceutical field for the prevention or treatment of central nervous system diseases such as parkinsonism, cerebral palsy and diabetic neuropathy; peripheral nervous system diseases such as diabetic peripheral neuropathy and traumatic nerve damage; diseases of the cardiovascular system such as stroke, ischaemia-reperfusion damage and intermittent claudication; and damage to the immune system in conditions of low oxygen tension.

The efficacy of the co-ordinated use according to the invention has been confirmed by various pharmacological tests, some of which are reported here below.

PHARMACOLOGICAL TESTS

I) Index of oxidative damage by evaluation of thiobarbituric acid reaction products (TBARS) produced by erythrocytes

Animal treatment

For this study 20 male Wistar rats aged 3 months were used. All animals were treated with a mixture of primary antioxidants (vitamin E 200 UI/kg and ascorbic acid 30 mg/kg) administered orally for 30 consecutive days. In a subgroup of 10 rats oral L-carnitine (50 mg/kg) was added to the basic treatment. The study was conducted according to the "open" experimental design and the choice of the subgroup of animals receiving combined antioxidant plus L-carnitine treatment was done using a random method.

Red blood cell preparation

At the end of the treatment, venous blood samples were taken in test tubes containing heparin. Leukocytes and platelets were removed via a chromatographic column containing cellulose and alpha-cellulose (1:1 w/w). The red blood cells were then washed three times with saline solution.

Incubation conditions

All incubations were done in an oscillating bath at 37°C. For experiments with intact cells, the red blood cells were washed one last time with Krebs incubation buffer (NaCl 120 mM; KCl 5 mM; $MgSO_4$ 1 mM; NaH_2PO_4 1 mM; sucrose 40 mM; glucose 5 mM; Tris-HCl 10 mM; pH 7.4) and resuspended in the same buffer at a haematocrit of 5%. The erythrocytes were treated with ter-butyl hydroperoxide (t-BOOH), a chemical agent capable of generating lipoperoxidative phenomena in the erythrocyte membrane, at a concentration of 2 mM. At the end of the treatment with t-BOOH, the erythrocytes were washed three times with incubation buffer. All washings were done at 4°C.

Determination of the oxidative damage index

The oxidative damage index chosen was the thiobarbituric acid reaction product (TBARS) index. For the determination and quantification of TBARS produced by the erythrocytes the method described by Tsun-Yee Chiu and Claster [*Methods in Haematology* Vol. 19, pp. 203-236. Churchill Livingstone, New York] was used.

The results are presented in **Figure 1**.

II) Index of oxidative stress by evaluation of the phosphatidylethanolamine/sphingomyelin ratio

Animals were treated and red blood cells prepared as in the previous test. The incubation conditions were

also identical to those used in the previous test.

Extraction and separation of phospholipids

The lipid components of the erythrocyte membrane were extracted according to the method described by Rose and Oklander [Rose, H.G. and Oklander, M.. *J Lipid Res*. (1965) **6**: 428-431]. To prevent oxidative phenomena butylhydroxytoluene (BHT 0.1%) was added to the extraction solvents. The lipid extract was dried under nitrogen flow and resuspended with toluene. For the separation of the individual phospholipid classes two-dimensional thin-layer chromatography was used [Rouser, G. et al., *Lipids* (1970) **5**: 494-496]. The phospholipid classes were highlighted with iodine vapours The individual phospholipid classes were identified by means of the use of standards. Determination of the phosphorus present in the individual phospholipid classes was done according to Bottcher [Bottcher. C.J.F. et al., *Anal. Chim. Acta* (1961) **24**: 203-208).

The results are presented in **Figure 2**.

III) Haemolysis in the course of oxidative stress

Animals were treated and red blood cells prepared as in the previous test. The incubation conditions were also identical to those used in the previous test.

Determination of haemolysis

Erythrocyte haemolysis was evaluated by measuring the amount of haemoglobin in the course of incubation with the oxidizing agent.

At the end of incubation, the erythrocytes were centrifuged and the buffy coat was used for the determination of haemoglobin released in the course of oxidative stress.

The haemoglobin determination was done spectrophotometrically according to the method described by Winterbourn C., *Handbook of Methods for Oxygen Radical Research* (ed. R. Greenwad) pp. 13-41, CRC Press, Boca Raton, 1985.

The results are presented in **Figure III**.

The addition of L-carnitine to the antioxidant mixture significantly reduces oxidative damage to the cell membrane. The quantity of TBARS was significantly reduced in erythrocytes obtained from the rats treated with L-carnitine and antioxidants compared to those treated with antioxidants alone (**Figure 1**). Following oxidative stress, the phosphatidylethanolamine/sphingomyelin ratio of the erythrocytes of animals treated with antioxidants alone was reduced to a greater extent than that of erythrocytes treated with antioxidants plus L-carnitine (**Figure 2**). Finally, haemolysis measured in the course of oxidative stress was significantly increased in the erythrocytes of animals treated with antioxidants alone compared to those treated with antioxidants plus L-carnitine (**Figure 3**).

An appropriate pharmaceutical composition in unit dosage form comprises approximately 0.3 to approximately 0.5 g of L-carnitine or an equivalent amount of alkanoyl L-carnitine or their pharmacologically acceptable salts and approximately 50 to approximately 2000 U/l or, preferably, approximately 300 to approximately 1000 U/l of vitamin E and/or approximately 50 to approximately 500 mg or, preferably, approximately 100 to approximately 300 mg of vitamin C.

The following non limiting examples show some compositions according to the present invention.

Examples

1) L-carnitine mg 500; Vit. E U/l 1000; Vit. C mg 300
2) acetyl L-carnitine mg 500; Vit. E U/l 1000; Vit. C mg 300
3) propionyl L-carnitine mg 500; Vit. E U/l 1000; Vit. C mg 300
4) isovaleryl L-carnitine mg 500; Vit. E U/l 1000; Vit. C mg 300
5) valeryl L-carnitine mg 500; Vit. E U/l 1000; Vit. C mg 300
6) butyryl-L-carnitine mg 500; Vit. E U/l 1000; Vit. C mg 300
7) L-carnitine mg 500; Vit. A mg 1000; Vit C. mg. 300
8) acetyl L-carnitine mg 500; Vit. A mg 1000; Vit C. mg. 300
9) propionyl L-carnitine mg 500; Vit. A mg 1000; Vit C. mg. 300
10)isovaleryl L-carnitine mg 500; Vit. A mg 1000; Vit C. mg. 300
11)valeryl L-carnitine mg 500; Vit. A mg 1000; Vit C. mg. 300
12)butyryl L-carnitine mg 500; Vit. A mg 1000; Vit C. mg. 300
13)L-carnitine mg 500; Vit. E U/l 1000; GSH mg 500
14)L-carnitine mg 500; Vit. E U/l 1000; Vit C. mg. 300; GSH mg 500
15)L-carnitine mg 500; Vit. A mg 100; Vit. C mg 300, selenium mg 40.

Claims

1. Use of L-carnitine, an alkanoyl L-carnitine wherein the alkanoyl group, straight or branched, has 2-6 carbon atoms or the pharmacologically acceptable salts thereof in combination and admixture with a lipophilic antioxidant and/or a hydrophilic antioxidant for preparing a medicament for the prevention and treatment of disturbances and diseases elicited by the oxidative stress brought about by oxygen free radicals.

2. The use of claim 1, wherein the aforesaid disturbances and diseases comprise central nervous system diseases such as parkinsonism, trauma, cerebral palsy, diabetic neuropathy and ageing;

peripheral nervous system diseases such as diabetic peripheral neuropathy and traumatic nerve damage; diseases of the cardiovascular system such as intermittent claudication, ischaemic-reperfuson damage and stroke; and immune system abnormalities in conditions of low oxygen tension.

3. The use of claims 1 or 2, wherein the alkanoyl L-carnitine is selected from acetyl-, propionyl-, butyryl, valeryl and isovaleryl L-carnitine.

4. The use of claims 1-3, wherein the lipophilic antioxidant is selected from vitamin E and vitamin A.

5. The use of claims 1-3, wherein the hydrophilic antioxidant is selected from vitamin C, glutathione (GSH) and selenium.

6. An orally, parenterally, rectally or transdermally administrable pharmaceutical composition for the prevention and treatment of disturbances and diseases elicited by the oxidative stress brought about by oxygen free radicals which comprise L-carnitine, an alkanoyl L-carnitine wherein the alkanoyl group, straight or branched, has 2-6 carbon atoms or the pharmacologically acceptable salts thereof in combination and admixture with a lipophilic antioxidant and/or a hydrophilic antioxidant, and a pharmacologycally acceptable excipient thereof.

7. The composition of claim 6 for the prevention and treatment of disturbances and diseases of the central nervous system such as parkinsonism, trauma, cerebral palsy, diabetic neuropathy and ageing; the peripheral nervous system diseases such as diabetic peripheral neuropathy and traumatic nerve damage; diseases of the cardiovascular system such as intermittent claudication, ischaemic-reperfuson damage and stroke; and immune system abnormalities in conditions of low oxygen tension.

8. The composition of claim 7, wherein the alkanoyl L-carnitine is selected from acetyl-, propionyl-, butyryl, valeryl and isovaleryl L-carnitine.

9. The composition of claims 7 and 8, wherein the lipophilic antioxidant is selected from vitamin E and vitamin A.

10. The compositon of claims 7 and 8, wherein the hydrophilic antioxidant is selected from vitamin C, glutathione (GSH) and selenium.

11. The composition of claim 9 in unit dosage form which comprises 0.3-0.5 g of L-carnitine or an alkanoyl L-carnitine and 50-2,000, preferably 300-1,000 U/l of vitamin E.

12. The composition of claim 10 in unit dosage form which comprises 0.3-0.5 g of L-carnitine or an alkanoyl L-carnitine and 300-500 mg of vitamin C.

13. The composition of any of the preceding claims which further comprises polyphenols, anthocyanins, anthocyanosides, mineral salts and vegetal fibers.

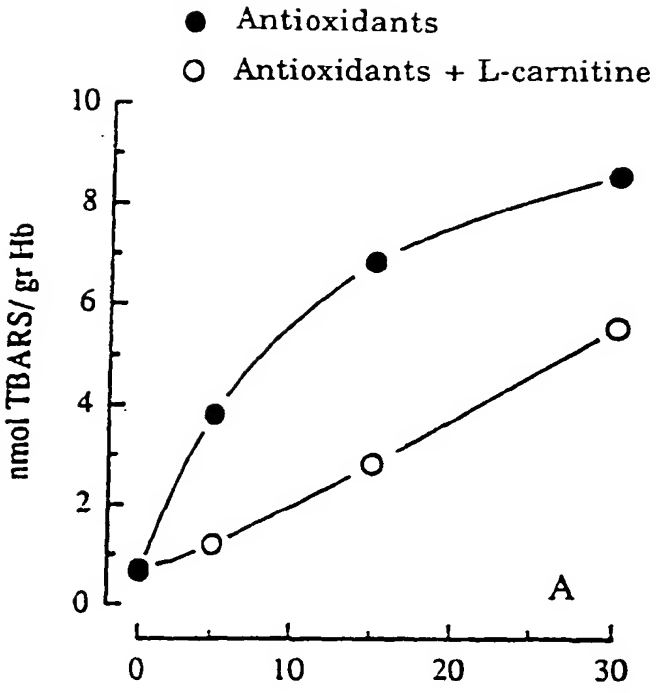

Figure 1

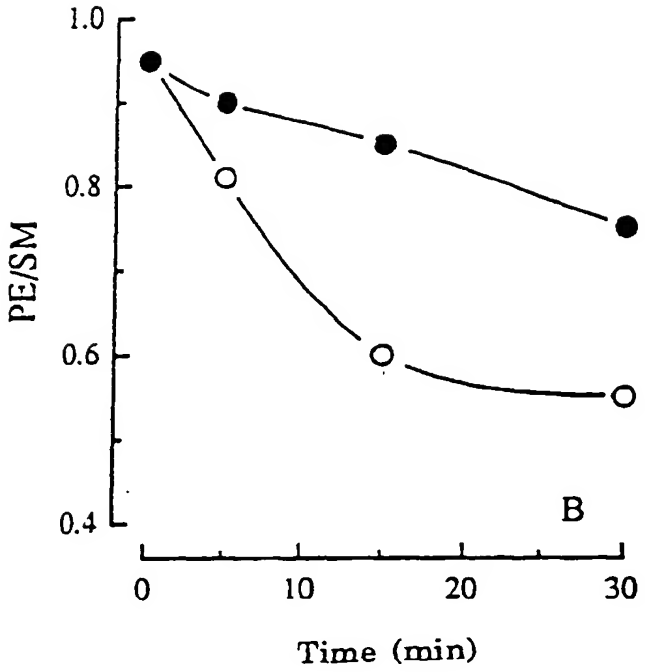

Figure 2

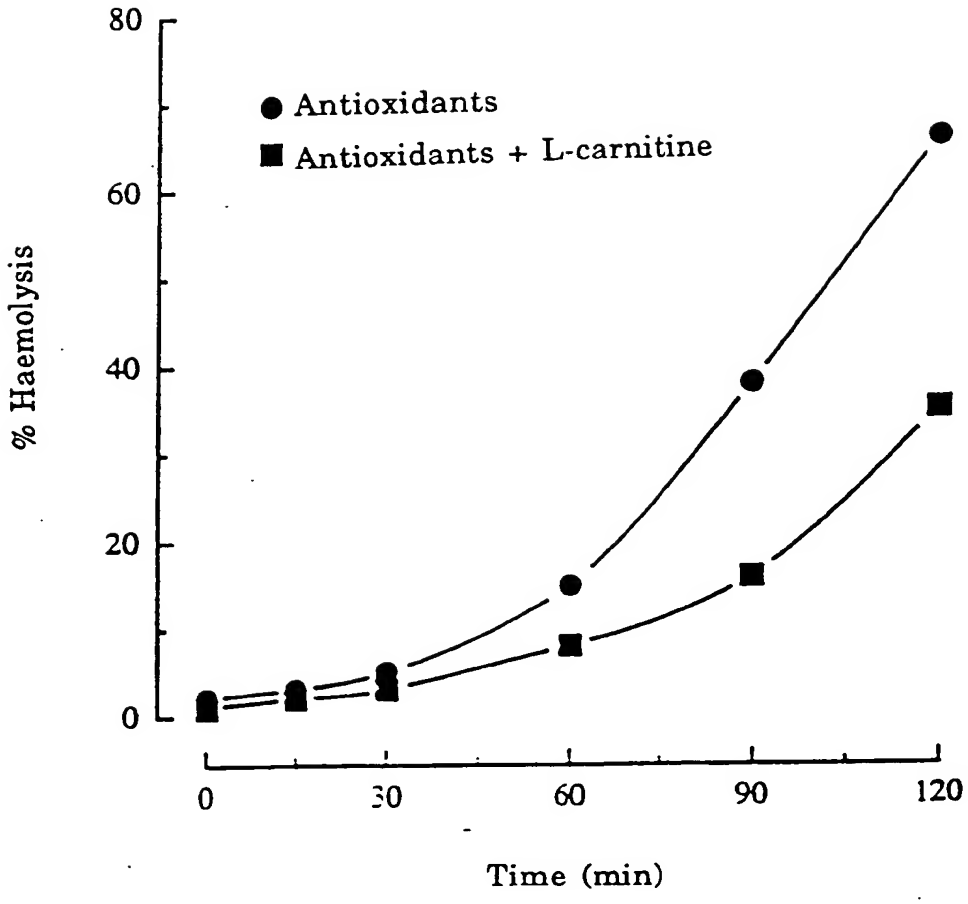

Figure 3

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EUROPEAN SEARCH REPORT

Application Number
EP 97 10 3727

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
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X	DE 43 35 454 A (P.SCHLEICHER) * claim 1 * ---	6,7, 9-11,13	
X	WO 94 02036 A (METAGENICS, INC.) * page 17 * ---	6,7,9, 10,13	
Y	DRUGS EXP. CLIN. RES., vol. 20, no. 5, 1994, pages 191-197, XP000654178 A.BERTELLI ET AL.: "L-Propionyl carnitne protects erythrocytes and low density lipoproteins against peroxidation" * abstract * * page 192, left-hand column, paragraph 2 * * page 196, left-hand column * ---	1-13	TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	27 June 1997	Tzschoppe, D

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

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Y	MED.SCI.RES., vol. 16, no. 13, 1988, pages 699-700, XP000654144 E.GEREMIA ET AL.: "Antioxidant action of acetyl-L-carnitine: in vitro study" * page 699, left-hand column * * page 700, left-hand column, paragraph 3 - right-hand column, paragraph 2 * ---	1-13	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
Y	MOL.CELL.BIOCHEM., vol. 88, no. 1-2, 1989, pages 161-168, XP000654126 R.FERRARI ET AL.: "Protective effect of propionyl-L-carnitine against ischaemia and reperfusion damage" * abstract * * page 161, left-hand column, paragraph 2 - right-hand column, paragraph 1 * * page 167, left-hand column, paragraph 4 * --- -/--	1-13	

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